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Adaptation of Microorganisms and Their Transport Systems to High Temperatures

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ABSTRACT. Growth of *Bacteria* and *Archaea* has been observed at temperatures up to 95 and 110°C, respectively. These thermophiles are adapted to environments of high temperature by changes in the membrane lipid composition, higher thermostabilities of the (membrane) proteins, higher turnover rates of the energy transducing enzymes, and/or the (exclusive) use of sodium-ions rather than protons as coupling ion in energy transduction. The proton permeability of the cytoplasmic membrane of bacteria and archaea was observed to increase with the temperature. This increased proton permeability limits the maximum temperature of growth of bacteria. Higher growth temperatures can be reached by an increased proton pumping activity by using the less permeable sodium ions as coupling ions or by changing the lipid composition of the cytoplasmic membrane. The Na^+/H^+/glutamate transport proteins of the thermophiles *Bacillus stearothermophilus* ($GltT_{Bs}$) and *Bacillus caldotenax* ($GltT_{Bc}$) were studied extensively. These transportproteins have unique features. Transport of L-glutamate occurs in symport with 1 Na^+ and 1 H^+ when the transport proteins are expressed in their natural environment. The sodium ion dependency of the GltT transporters of these *Bacillus* strains was found to increase with temperature. However, when the GltT proteins are expressed in the mesophile *Escherichia coli*, electrogenic symport of L-glutamate occurs with $\geq$ 2 H^+. These observations suggest that the conformation of the transport proteins in the *E. coli* and the *Bacillus* membranes differs, and that the conformation influences the coupling ion selectivity. The Na^+/H^+/glutamate transport proteins of *B. stearothermophilus* ($GltT_{Bs}$) and *B. caldotenax* ($GltT_{Bc}$) are homologous to transport systems of glutamate and structurally related compounds from mesophilic organisms. Both sodium, as well as proton coupled transporters, belong to this family of carboxylate transporters (FCT). COMP BIOCHEM PHYSIOL 118A;3:423–428, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Thermophile, thermostability, membranes, cation-selectivity, glutamate transport

INTRODUCTION

Bacteria and *Archaea* comprise a thermal span far greater than that of the third domain, the *Eucarya* (the phylogenetical subdivision as proposed by Woese *et al.* [30] is used). *Bacteria* and *Archaea* can be classified into psychrophiles, mesophiles, and thermophiles with optimal growth temperature ranges of 0–20, 10–50, and 40–110°C, respectively. The latter group can be subdivided into moderate thermophiles, with optimal growth in the range of 55–65°C, facultative thermophiles that are able to grow over a wide temperature span, and extreme thermophiles (hyperthermophiles) that grow only above the "thermophile boundary" of 55–65°C (2,3). Some hyperthermophiles belonging to the *Bacteria* have an upper growth temperature of about 95°C, whereas growth of some *Archaea* has been observed at temperatures as high as 110°C. So far, it is not clear what the upper temperature limit of growth is, or what the limiting factor is for growth at high temperatures. Despite several adaptations to growth at elevated temperatures, thermophiles often exhibit lower growth yields and higher maintenance-energy requirements than mesophiles (8,14). Investigations of the mechanism of adaptation to life at high temperatures is likely to reveal important features of protein structure, protein-protein interactions, membrane structure, and lipid-protein interactions that are not found in mesophilic organisms. For instance, enzymes of thermophiles need to be intrinsically stable at the elevated temperatures of growth. In this paper the thermoadaptation mechanisms of membrane proteins, the membrane barrier and the bioenergetics of the cell will be discussed.

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ADAPTATION OF MICROORGANISMS TO HIGH TEMPERATURES

Thermophiles (*Bacteria* and *Archaea*) can respond or adapt to thermal stress by changing the lipid composition and the

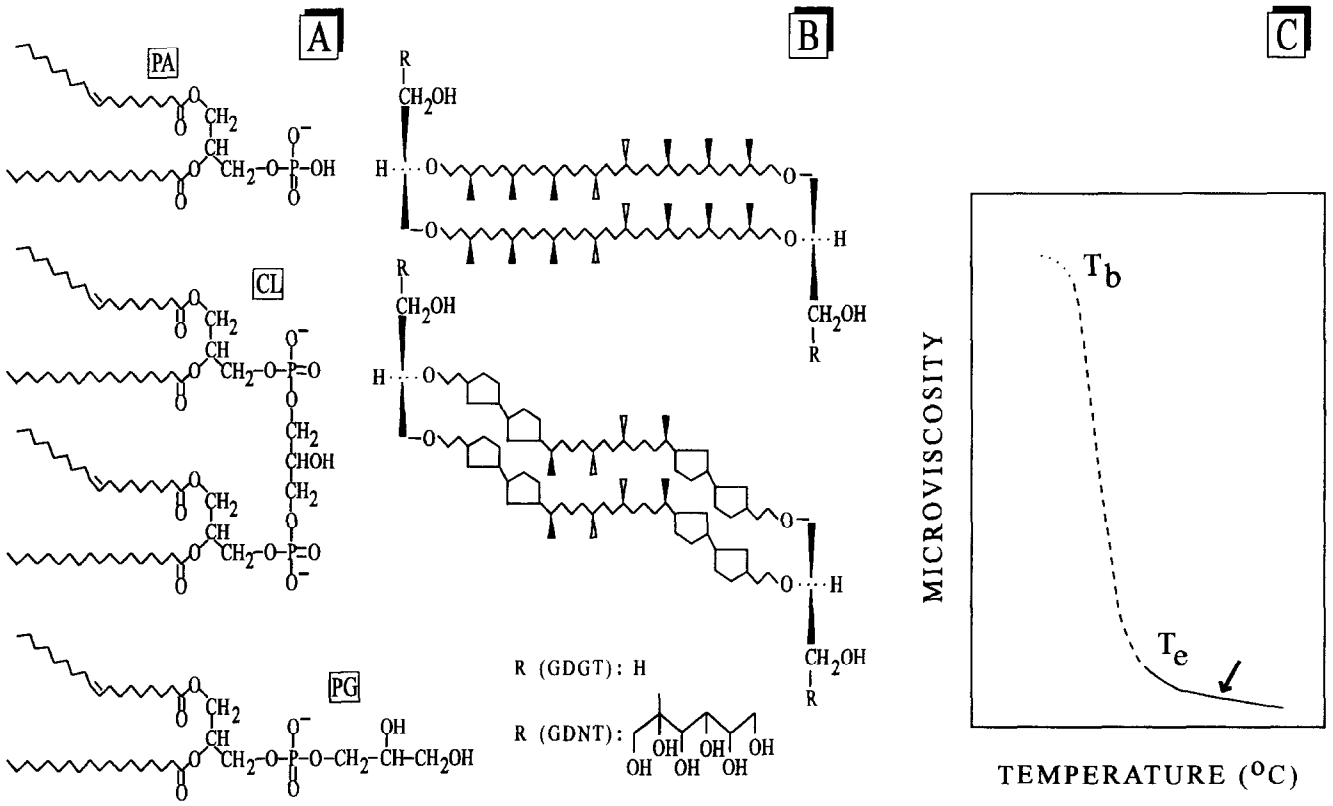

FIG. 1. Structure of some membrane lipids of *Bacteria* (A) and *Archaea* (B); and the effect of temperature on viscosity of membranes (adapted from [5]) (C). Abbreviations: CL, cardiolipin (diphosphatidylglycerol); GDGT, glycerol-dialkyl-glycerol tetraether; GDNT, glycerol-dialkyl-glycerol tetraether; PA, phosphatidic acid; PG, phosphatidylglycerol; R, side chain; T_b and T_e, begin and end of transition phase, respectively. Symbols: dotted line, gel phase; dashed line, transition phase; solid line, liquid crystalline phase of a membrane; arrow, putative optimal growth temperature.

energy transducing and ion permeability properties of the cytoplasmic membrane.

Lipid Mono/bilayer and Lipid Protein Interaction

LIPID MONO/BILAYER. The cytoplasmic membrane plays a vital role as a selective barrier for the entry and exit of solutes into or out of the cell. Thermophiles differ markedly from mesophiles with respect to the structure of the (cytoplasmic) membrane. The cytoplasmic membrane of *Bacteria* consists of a lipid bilayer of mainly diacyl-glycerol diesters that have a 1,2-*sn* stereochemistry (Fig. 1A and B). In contrast, characteristic of *Archaea* is the presence of ether lipids in the cytoplasmic membranes (Fig. 1A and B). The membranes of most *Archaea* consist predominantly of diethers (counterpart of the typical bacterial "diesters") while in thermophilic *Archaea* tetraethers which form a monolayer are found (4). Membrane proteins are embedded in these lipid layers. The lipid-(mono/bi) layers have an asymmetric organization, and lipids can be distributed asymmetrically over the two leaves of the membrane.

LIPID-PROTEIN INTERACTION. The lipid-protein interaction is such that membrane proteins are bound to the lipid-layer via electrostatic and/or hydrophobic interactions, and in some cases via covalent linkage (lipoproteins) (24). Lipids in the cytoplasmic membrane can be classified as bulk, annular, or bound (11): 1) The *bulk* lipids form the matrix in which the proteins are embedded. These lipids determine mainly the "membrane fluidity" (see below). 2) The *annular* lipids comprise those lipids that cover the surface of the membrane proteins. These lipids can play an important role in stabilizing the native conformation of the enzymes. A preference has been observed of membrane proteins to interact with negatively charged lipids like cardiolipin (CL; diphosphatidyl glycerol) and phosphatidyl glycerol (PG). 3) *Bound* lipids are those lipids that bind specifically and with high affinity to certain membrane proteins.

In general, lipids affect enzymes by influencing the catalytic activity, tertiary structure, movement, and aggregation state (1,11). For example, a fatty acid acyl chain carbon number of 18 was found to be optimal for activity of the *Lactococcus lactis* branched-chain amino acid transporter, indicating the importance of matching of the lipid molecules with the hydrophobic thickness of the carrier protein (10). The lipid-protein interactions may also affect thermostability of membrane proteins.

Membrane Fluidity

MEMBRANE FLUIDITY. Membrane fluidity (inverse of membrane viscosity) partly comprises the effects of lateral and rotational diffusion of lipids, as well as rotation of lipids around single carbon-carbon bonds. The physical state of membranes can be in a gel, transition, or liquid-crystalline phase. The occurrence of these phases in (bacterial) membranes can be observed in a plot of viscosity versus temperature (Fig. 1C). Several studies have shown that integral membrane proteins function optimally in the liquid-crystalline phase.

HOMEOVISCOUS ADAPTATION. Whereas in *Eucarya* membrane fluidity is mainly modulated by varying the phospholipid/cholesterol ratio, this modulation is more complex in *Bacteria* and *Archaea*. *Bacteria* alter their lipid composition such that the membrane fluidity is relatively constant at different growth temperatures. This process is referred to as "homeoviscous adaptation" (22). The adaptation is such that the upper temperature of the phase transition from gel to liquid-crystalline phase (T_c, Fig. 1C). is below the growth temperature. The phase transition temperature shifts to higher temperatures upon growth at increasing temperatures, which means that the curve depicted in Fig. 1C shifts to the right. Thermoadaptation of the lipid-layer can involve a.o., an increase in acyl-chain length, saturation, branching (or altering anteiso to *iso* branching), and/or cyclization of the fatty acids upon increasing the temperature (19,21,25). In general, the fraction of lipids with a low melting point decreases on increasing growth temperatures as a result of these changes. Furthermore, the temperature of phase transition of lipids is also affected by factors such as ionization of the lipids, the ratio of lipids vs. other membrane components (e.g., proteins) in the membrane, and the presence of organic ions (e.g., alcohols and free fatty acids).

In *Archaea*, an increase in cyclization in a response to increasing temperature has been observed; a single phytanyl chain of the di- or tetraethers can contain 0 to 4 cyclopentane groups (9). Unsaturated etherlipids have also been observed as an adaption to lower temperature.

HOMEOPHASIC THEORY. The "homeophasic theory" (16) has been postulated as an alternative to the "homeoviscous adaptation" theory. It emphasizes that maintenance of the liquid-crystalline phase is more important than an absolute value of membrane fluidity. Also, temperature-dependent changes in lipid composition in *Bacteria* are meant to prevent the transition from the liquid-crystalline phase to nonbilayer phases. The regulatory mechanism is based on lipid polymorphism. According to their shape, lipids can be grouped into the following classes: cone, inverted cone, and cylindrical. These lipids can form micelles, inverted micelles and bilayers, respectively. By altering the ratio of these differently formed lipids the liquid-crystalline phase can be maintained at elevated temperatures as has been observed in *Acholeplasma laidlawii* (16). Thermoadaptation, therefore, may involve both homeoviscous and homeophasic adaptation.

Efficiency of Energy Transduction and Proton Leakage

Thermophiles can grow at elevated temperatures due to thermostable/active enzymes and structural adaptations of the membranes. However, often a low growth yield and high maintenance requirement is observed. These latter observations may be explained by an increased ion-permeability of the membrane at high temperatures (5,7,25,31). Consequently, thermophiles will have to invest relatively more metabolic energy in generating an ion motive force than mesophiles. A number of adaptations that relate to the increased H^+-permeability of the membranes at elevated temperatures have been described.

LIPIDS AND PROTON PERMEABILITY. Liposomes have been prepared from lipids of the psychrophile *Psychrobacter* sp. (*Micrococcus cryophilus*, [T_{opt} of 21°C]), the mesophile *E. coli* (T_{opt} of 37°C), and the thermophiles *B. stearothermophilus* (T_{opt} of 63°C), *Thermatoga maritima* (T_{opt} of 80°C), and *S. acidocaldarius* (T_{opt} of 85°C). In all liposomes the H^+-permeability increased with temperature. However, the higher the growth temperature of the organism from which the lipids were derived, the higher the temperature at which the liposomes became very leaky for H^+ (7,29). These data suggest that a low H^+-permeability of the membranes is important for growth at high temperature. The lower H^+ permeability of the membranes of thermophilic *Archaea* (e.g., *S. acidocaldarius*) explains why the organism can grow at higher temperatures than *Bacteria*.

SODIUM CYCLE. The cytoplasmic membrane is much less permeable for sodium ions then for protons. Because of the high H^+-permeability, particularly at elevated temperatures, the use of a sodium motive force (Δs) can be of energetic advantage above a proton motive force (Δp). In accordance with these observations, sodium-ions have been found to play an important role in bioenergetics. In aerobic organisms such as *B. stearothermophilus* Na-ions are the main coupling ions in secondary solute transport processes, while protons play an essential role in ATP-synthesis. In anaerobes such as *Clostridium fervidus*, sodium-ions are found to be the only coupling ions in energy-transduction. In this way, the anaerobic organism can reduce loss of metabolic energy by leakage processes. However, a consequence of not using H^+ as coupling ions is that the organism cannot control its internal pH and, therefore, can only grow around neutral pH (23).

HIGH TURNOVER BY REDOX CONVERTING ENZYMES. To counteract the high H^+-permeability of the membranes, aerobic bacteria such as *B. stearothermophilus* have extremely high turnover rates of the proton pumping respiratory chain at its growth temperature (5).

As a result, the organism is able to generate a high proton

TABLE 1. Amino acid identity (similarity) between pairs of proteins

Protein[a]	*ion(s)*[b]	% Amino Acid Identity With:					
		$GluC_{Ck}$	$GluC_{Hs}$	$GluB_{Hs}$	$GluA_{Rn}$	$GluA_{Hs}$	
		100	91.2	49.8	51.9	51.3	$GluC_{Ck}$
			100	50.1	51.8	51.1	$GluC_{Hs}$
				100	48.1	47.1	$GluB_{Hs}$
$GltP_{Bsu}$	$\geq 2H^+$	100			100	96.9	$GluA_{Rn}$
$GltT_{Bs}$	$1\ Na^+ + 1H^+$	44.8	100			100	$GluA_{Hs}$
$GltP_{Ec}$	$\geq 2H^+$	43.6	60.1	100			
$DctA_{Rm}$		35.7	38.7	36.4	100		
$AscT1_{Hs}$	$\geq 2Na^+$	24.8	29.7	27.0	23.4	100	
$SatT_{Hs}$	$\geq 2Na^+$	23.9	26.1	23.3	22.5	93.6	100
$GluA_{Hs}$	$\geq 2Na^+$	24.6	32.5	26.5	26.5	40.6	39.7
$GluA_{Rn}$	$\geq 2Na^+$	24.8	31.6	27.9	25.4	40.8	39.1
$GluB_{Hs}$	$\geq 2Na^+$	24.3	29.9	27.2	25.4	37.8	36.9
$GluC_{Hs}$	$\geq 2Na^+$	26.0	29.7	28.2	22.7	32.0	31.2
$GluC_{Ck}$	$\geq 2Na^+$	26.5	28.5	27.2	24.9	34.0	32.6
		$GltP_{Bsu}$	$GltT_{Bs}$	$GltP_{Ec}$	$DctA_{Rm}$	$AscT_{Hs}$	$SatT_{Hs}$

Abbreviations [Embl Databank Accession Number]: $GltP_{Bsu}$, *Bacillus subtilis* H^+/Glu symport protein [U15147]; $GltT_{Bs}$ and $GltT_{Bs}$, *B. stearothermophilus*, and *B. caldotenax* Na^+/H^+/Glu symport protein [M86508 and M86509, respectively]; $GltP_{Ec}$, *Escherichia coli* H^+/Glu symport protein [M84805]; $DctA_{Rm}$, *R. meliloti* C4-dicarboxylate transport protein [J03683, M26399 and M26531, respectively]; $AscT1_{Hs}$, *H. sapiens* Ala/Ser/Cys/Thr transporter [L14595]; $SatT_{Hs}$, *H. sapiens* neutral amino acid transporter [L19444]; $GluA_{Hs}$, $GluB_{Hs}$, $GluC_{Hs}$, *H. sapiens* excitatory amino acid transporter 1, 2 and 3, respectively. [U03504, D26443, L19158; U03505 and U03506, respectively]; $GluB2_{Hs}$, *H. sapiens* Glu transporter [Z32517]; $GluA_{Rn}$ (is GLAST or GLUT-1), *R. norvegicus* Glu/Asp transporter [X63744 and S59158, respectively]; $GluC_{Ck}$ (is EAAC1), *O. cuniculus* Glu transporter L12411].

The numbers indicate how many ions are symported with 1 molecule L-glutamate. The stoichiometry of ion/glutamate transporters listed with "$\geq$ 2 N" listed with "$\geq$ 2 Na^+" has to be confirmed.

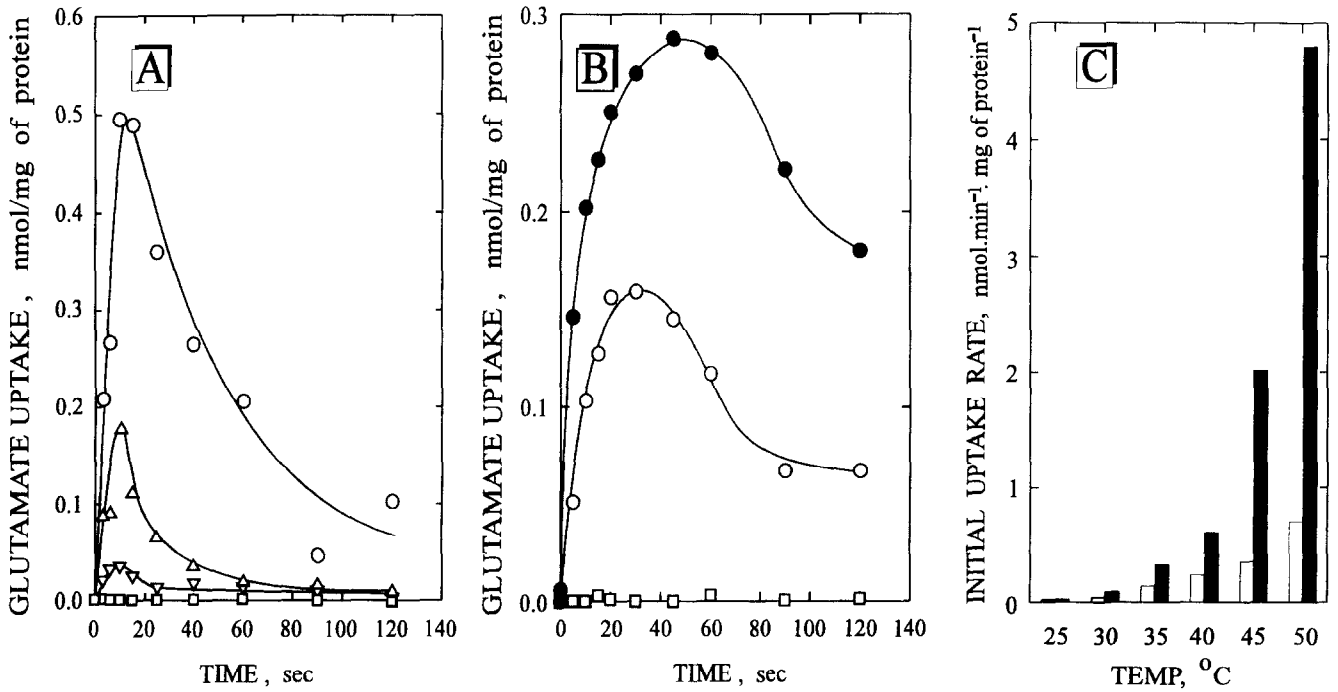

FIG. 2. Uptake of L-glutamate in membrane vesicles of *Escherichia coli* ECOMUT1/pGBT112 ($GltT_{Bs}$) (A) and *Bacillus stearothermophilus* ($GltT_{Bs}$) (B), and temperature-dependent sodium-stimulation of L-glutamate in membrane vesicles of *B. stearothermophilus* ($GltT_{Bs}$) (C), driven by artificially imposed ion gradients. L-Glutamate uptake was performed in the presence of an artifically generated Δp (○), $\Delta p + \Delta pNa$ (●), ΔpH (△) or $\Delta\Psi$ (▽) (see [28] for experimental details). This assay temperature was 37 or 45°C, or varied in Fig. 3A, B, and C, respectively. The effect of temperature on the initial uptake rate in the presence (shaded bars) and absence (open bars) of Na^+ ions (ΔpNa) is shown. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (□).

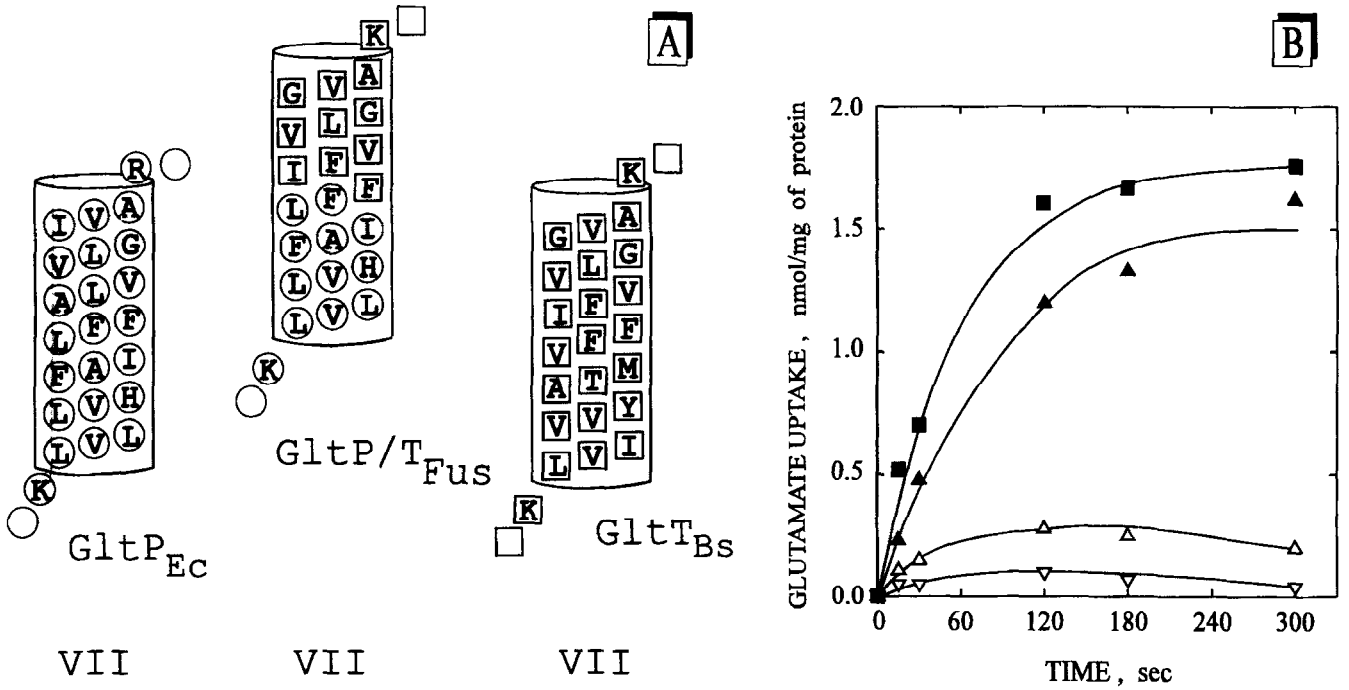

FIG. 3. Fusion point (in putative α-helix VII) between GltP$_{Ec}$ and GltT$_{Bs}$ in the hybrid GltP/T$_{Fus}$ (A), and uptake of L-glutamate in membrane vesicles of *Escherichia coli* BK9MDG/pFUS101 (GltP/T$_{Fus}$) and *E. coli* BK9MDG/pUC18 (B). Uptake of L-glutamate was measured at 37°C by diluting membrane vesicles of *E. coli* BK9MDG/pFUS101 (GltP/T$_{Fus}$) (▲), *E. coli* BK9MDG/pGBT521 (GltP$_{Ec}$) (■) and BK9MDG/pUC18 (△) into oxygen saturated 50 mM potassium phosphate, pH 6.0, 5 mM MgSO$_4$, 10 mM K-asc and 100 μM PMS. After 1 min of incubation. L-[^{14}C]-glutamate (1.9 μM) was added. Control experiments were performed by diluting the membrane vesicles into buffer without electron donor (▽).

motive force despite the high proton permeability of the membrane.

In conclusion, energy transduction in the cytoplasmic membrane at elevated temperatures can be realized by: 1) an adjusted membrane composition (e.g., as found in archaeal membranes), 2) the use of sodium as a coupling ion, and/or 3) high rates of proton pumping by respiration.

TRANSPORT SYSTEMS OF THERMOPHILES

Bacteria and *Archaea* have been described in which the bioenergetic cationic cycle involves the exclusive use of Na^+ or H^+, or those in which Na^+ or H^+ are used simultaneously (6,15,23). The metabolic energy conserved in sodium and/or proton electrochemical gradients can be used to drive energy-consuming processes such as secondary solute transport, ATP synthesis, and motility. The enzymes (like primary and secondary transport systems that generate and maintain these ion and solute concentration gradients are located in the cytoplasmic membranes (17,18,23). Secondary transporters of L-glutamate from thermophilic bacilli have been extensively studied.

Secondary Transport Systems of Thermophiles and Coupling Ions

The genes encoding the glutamate transporters of the thermophiles *B. stearothermophilus* (gltT$_{Bs}$), *B. caldotenax* (gltT$_{Bc}$), and *Bacillus* IS1 (gltT$_{Bi}$), and of the mesophile *B. subtilis* (gltT$_{Bsu}$) have been cloned, characterized, and functionally expressed in *E. coli* (26–28). These transport proteins are all homologous to the proton-glutamate symporter of *E. coli* K12 (GltP$_{EcK12}$; [26] [Table 1]). These glutamate transporters also have similar substrate specificities, but differ with respect to the coupling ion (28). Studies in membrane vesicles of *B. stearothermophilus* and *B. caldotenax* indicated a Na^+/H^+/L-glutamate symport for both GltT$_{Bs}$ and GltT$_{Bc}$ (Fig. 2B). Surprisingly, however, when the proteins are expressed in *E. coli*, GltT$_{Bs}$ and GltT$_{Bc}$ catalyze electrogenic symport of L-glutamate with $\geq 2H^+$ (Fig. 2A). Furthermore, the sodium ion dependencies of the GltT transporters in the *Bacillus* strains increase with temperature (Fig. 2C). These observations suggest that the conformation of the transport proteins influences the coupling ion selectivity (28).

The glutamate transporters are also homologous to a number of other proteins that all transport one or more of the structurally related compounds glutamate, aspartate, fumarate, malate, and/or succinate (12,13,20,27). This family of carboxylate transporters (FCT) comprises sodium, as well as proton, coupled transporters (27; Table 1).

Hydropathy profiling and multiple alignment of the FCT members suggests that each of the proteins spans the cytoplasmic membrane 12 times with both the amino- and carboxy-terminal region on the inside (27).

To dissect the domain structure and function, it would be useful to generate fusions between the Na^+-dependent $GltT_{Bs}$ and Na^+-independent $GltP_{Ec}$ transport proteins. Random fusions were made between the Na^+-dependent $GltT_{Bs}$ and Na^+-independent $GltP_{Ec}$. A functional hybrid protein, composed of the *N*-terminal region of the $GltP_{Ec}$ (amino acid residues 1 to 243) and the C-terminal region of $GltT_{Bs}$ (residues 234 to 421) ($GltP/T_{Fus}$; Fig 3A), was constructed, and found to catalyze H^+-, but not Na^+-dependent, transport (Tolner *et al.*, unpublished). The hybrid protein is highly active when expressed in *E. coli* BK9MDG (Fig. 3B), which indicates that the tertiary structures of $GltT_{Bs}$ and $GltP_{Ec}$ are very similar. The L-glutamate transporters of *B. stearothermophilus* has been solubilised, purified, and functionally reconstituted in liposomes (D.J. Slotboom, unpublished). Detailed studies on this reconstituted transporter will reveal the influence of the lipid environment on the cation-specificity.

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